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Effects of 5-fluorouracil on exocrine glands

III. Fine structure of Brunner's glands of rats

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INTRODUCTION

Five-fluorouracil (FUR), a pyrimidine analogue, has found its place in cancer chemotherapy, especially in the early treatment of skin cancers and more recently in treating malignant growths elsewhere. It has been shown in previous experiments with bacteria that FUR is extensively incorporated into the bacterial RNA, substituting for uracil, and therefore has profound effects on protein and RNA syntheses (Horowitz & Chargaff, 1959; Horowitz, Saukkonen & Chargaff, 1958, 1960). Biochemical studies on micro-organisms have shown that FUR, by producing 'fraudulent' RNA and ribosomes, leads eventually to defective protein synthesis (Aronson, 1961; Kempner & Miller, 1963; Hignett, 1964). In addition, a number of investigators have shown that it inhibits the synthesis of DNA (Heidelberger, 1965, 1967; Cherry, Chroboczek, Carpenter & Richmond, 1965). In causing tumor repression, its inhibition of thymidylate synthetase is probably more important than its effects on other biochemical steps (Heidelberger, 1967). Clinically, it has been reported that breast, large intestine and hepatocellular tumours respond the most favourably (Kennedy & Theologides, 1961).

To be effective in cancer chemotherapy FUR, like other antimetabolites, has to be used in highly toxic doses, and its toxic effects are most marked in tissues having rapid turnover, e.g. bone marrow, skin and gastrointestinal mucosa.

Brunner's glands, first mentioned by Wepfer (1679), are only found in mammals (Middledorf, 1846; Kuczynske, 1890; Bensley, 1903; Carleton, 1935; Elias, 1947; Florey, 1955). In species reported to date, with the exception of monotremes, the glands lie mainly in the submucosa of the duodenum and their ducts penetrate the muscularis mucosae to empty into the crypts of Lieberkuhn. Kuczynske (1890) reported that the glands were short in carnivores, intermediate in omnivores, and long in herbivores.

In the rat, the main bulk of the glands lies at the pyloroduodenal junction as a comma-shaped mass with a narrow tapering tail extending into the duodenum. The glands are tubulo-alveolar, and their structure and nuclear polarity vary with functional activity (Bensley, 1903). In general, the glands are lined by typical mucous secreting cells. However, Tschassownikow (1927) noticed another type of cell interspersed between the typical mucous cells which were conical in shape and had a dark, granular cytoplasm.

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The ultrastructure of the gland has been studied in many different animals, including the cat (Moe, 1960), guinea-pig (Cochrane *et al.* 1964), mouse (Friend, 1965), rat and rabbit (Martin, 1954; Leeson & Leeson, 1966, 1967), man (Leeson & Leeson, 1968), opposum (Krause & Leeson, 1969*a*, *b*), echidna and platypus (Krause, 1970, 1971). These studies have described the presence of both mucous and serous cells.

In light of the above, the present study was conceived (1) to explore further the ultrastructural details of Brunner's gland of the rat and (2) to study the effects of FUR on its acinar cells.

MATERIALS AND METHODS

Experiments

Eighteen adult male Sprague–Dawley rats weighing 200–250 g were kept in an air-conditioned animal quarter in the Department of Anatomy at the University of Michigan, and fed Purina rat chow and water *ad libitum*. At the beginning of the experiment rats were placed in individual cages and three rats of similar weights were grouped. One of the three was given a single injection of 50 mg/kg body weight of FUR per day for 3 days. This is a dose that had been determined to be sublethal in a preliminary dose–response study.

The experimental animal was pair-fed with one of the remaining two, while the last one of the group was given food *ad libitum* and designated as normal. Thus, the control rats indicated in this article will refer to those pair-fed control rats which received the amount of diet consumed by their experimental pair. Rats were kept for 5 additional days after the last FUR injection. Eighteen hours before killing, food was withdrawn from all rats. The animals were killed under anaesthesia with an intraperitoneal injection of 3·5% chloral hydrate (1 ml/100 g body weight) and perfused through the heart with fixative made up of 1·4% glutaraldehyde (TAAB Laboratories, Emmer Green, Reading, England), 1% paraformaldehyde and 0·025% $CaCl_2$ in 0·1 M cacodylate buffer at pH 7·4. Preliminary experiments had indicated that Brunner's glands fixed well after about 100–150 ml of the perfusate had been used.

Preparation of tissues for electron microscopy

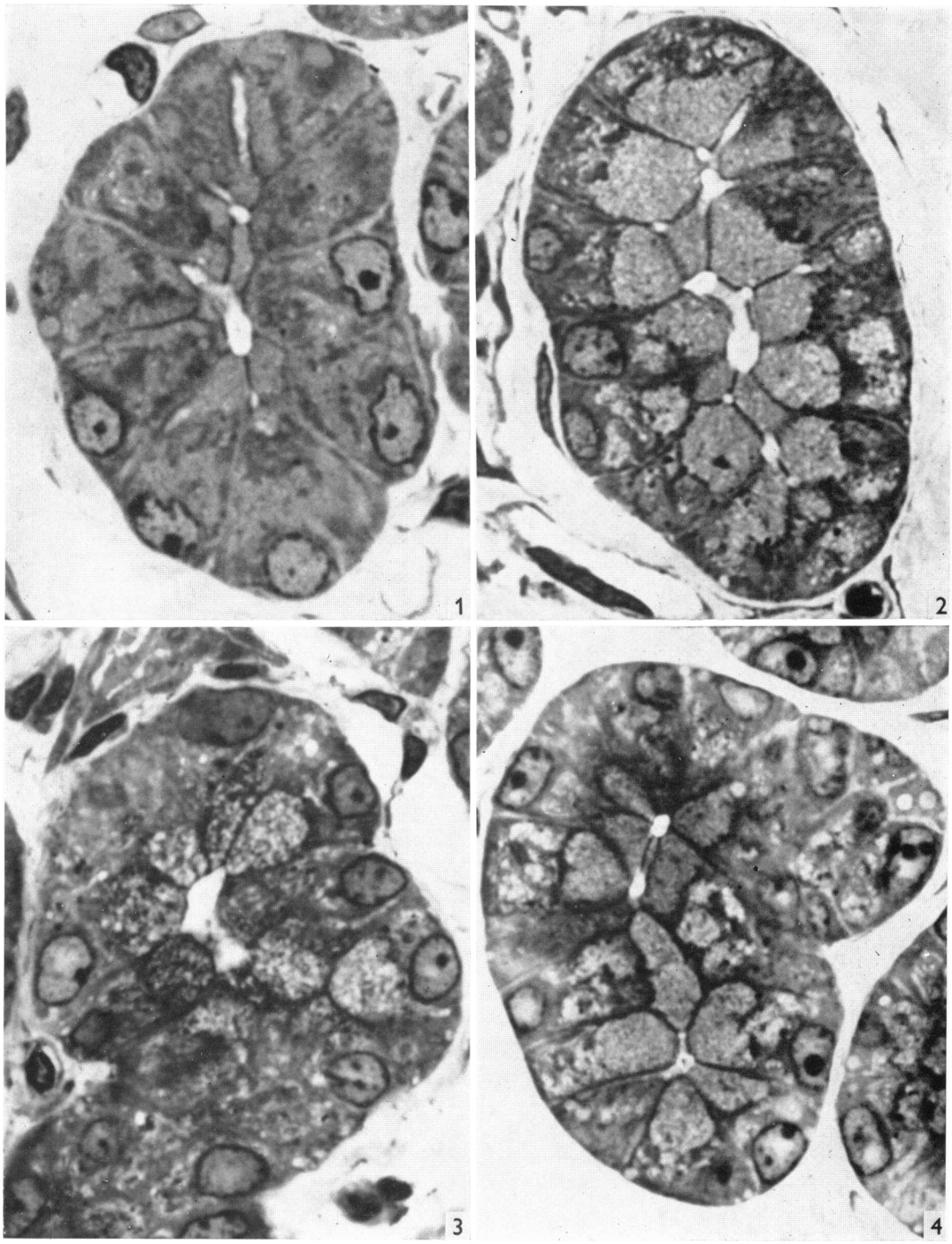
The first 2 cm of the small intestine was removed and split into two halves by cutting longitudinally along the mesenteric and anti-mesenteric borders. One half of the duodenum was transversely cut into thin (0·5 by 2·0 mm) slices for routine electron microscopic preparation. The second half of the tissue was prepared for electron microscopic cytochemistry as described later. The pieces of tissue were washed in 0·1 M sodium cacodylate buffer with 7·5% sucrose for 15–30 minutes and then post-fixed

Fig. 1. An acinus from the normal rat, showing few granules, and basophilic regions separated by a negative image of the Golgi apparatus. Toluidine blue. × 800.

Fig. 2. An acinus from the normal rat, showing numerous apical granules. The Golgi complex also contains granular material. Toluidine blue. × 800.

Fig. 3. An acinus from the control pair-fed animal. Cells are more irregular with varying granule contents. Note the frothy appearance of granules. Toluidine blue. × 800.

Fig. 4. An acinus from the FUR-treated animal. Note the more circumscribed appearance of the Golgi complex, and cytoplasmic lipid droplets. Toluidine blue. × 800.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in 1% OsO_4 with 0·1 M sodium cacodylate buffer for 1½ hours. The tissue was thereafter washed twice in 0·1 M sodium cacodylate buffer with sucrose and left overnight in the same buffer prior to dehydration and embedding in a mixture of Epon on the following day (Luft, 1961).

Sections 1 μm in thickness were made on an LKB ultrotome with glass knives and stained with toluidine blue to localize proper areas of the gland for observation. From selected regions ultrathin sections of up to 800 nm were made either with glass or diamond knives on the same ultramicrotome. The sections were collected on 300 mesh copper grids, double stained with saturated uranyl acetate and lead citrate (Reynolds, 1963). The sections were then examined with a Hitachi HS-8 electron microscope.

Enzyme cytochemical preparation of tissues

The remaining half of the tissue to be used for cytochemical studies was diced into the smallest possible sizes with single-edged razor blades while immersed in the chilled fixative. Following this, they were thoroughly washed in 0·1 M cacodylate buffer with sucrose and left overnight in the same buffer at 4 °C. Prior to incubation in the appropriate mixture the tissues were washed with three changes of the buffer to be used for different enzyme activities.

For demonstration of uridine diphosphatase (UDPase) and thiamine pyrophosphatase (TPPase) activities the tissues were washed in tris-maleate buffer and incubated in the Novikoff and Goldfischer medium with 5% sucrose (Novikoff *et al.* 1971). On the basis of our preliminary data, the amount of substrate was doubled. The incubation was carried out for 60 minutes at 37 °C with a change of incubation medium after 30 minutes, and was followed by three washings of the tissue with the same buffer prior to embedding.

For demonstration of acid phosphatase (AcPase) activity tissues were incubated in a modified Gomori medium in which disodium glycerophosphate was replaced by 5′-cytidine monophosphate (CMP) and sucrose added (Novikoff *et al.* 1971). The embedding of tissues incubated for demonstration of TPPase and AcPase activities was done in the manner similar to the routine procedure described previously.

RESULTS

Light microscopy

One μm sections of the Brunner's gland taken from normal rats that were starved for 18 hours before killing demonstrated a range of variation in its structure which appeared to reflect different stages in its secretory cycle. There were secretory portions of the gland which contained practically no granules (Fig. 1). These cells had a basophilia which was divided between the basal and apical regions of cytoplasm by a supranuclear Golgi complex. The nucleus was basally located, having a somewhat jagged contour and contained prominent nucleoli.

The other extreme of the normal acinar structure was characterized by the presence of finely granular secretory material filling the entire apical cytoplasm (Fig. 2). In such acini the Golgi region was clearly demonstrated as irregular patches of light areas corresponding in staining quality to that of secretory granules. The nuclei of these cells had some less prominent nucleoli.

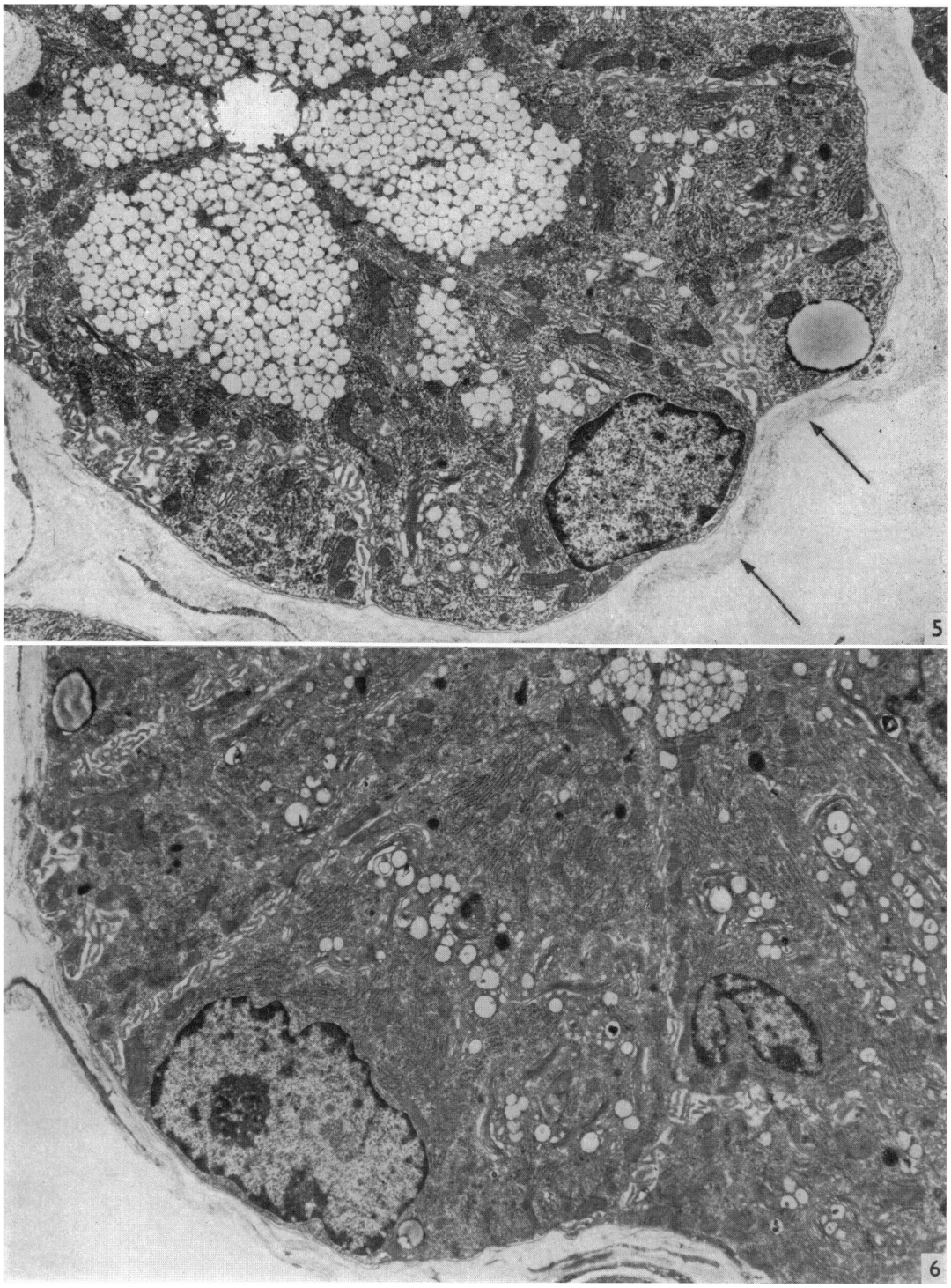


Fig. 5. A portion of an acinus from a normal rat, showing abundance of secretory granules that fill up the apical half of the cells (storage phase). Intercellular canaliculi are present between basal regions of the neighbouring cells. × 6000.

Fig. 6. A portion of the acinus from a normal rat, showing only a small amount of apical granules. Supranuclear Golgi complexes divide the RER into two regions. × 6000.

The histological appearances in the pair-fed control rats differed in a number of ways from those of normal animals. Cells were more irregular in shape and the apical cytoplasm contained secretory granules that were pale and appeared frothy. The Golgi region was more diffuse and contained less clear aggregations of secretory materials as compared with the normal gland. The nucleus was basally located and had small nucleoli (Fig. 3).

The glands from FUR-treated rats had areas of marked changes that alternated with comparatively normal-looking areas (Fig. 4). The cells were small in size and the secretory granules appeared to fill the apical third to half of the cells. Golgi regions appeared to be smaller than in controls. A number of cytoplasmic vacuoles and granules that were stained bluish green were noted.

Electron microscopic observations

Normal gland cells

The acinar cells of Brunner's glands from normal animals after 18 hours of starvation showed differences in the amount of secretory granules present in the apical cytoplasm (Figs. 5, 6). The acinar cells rested on a continuous basal lamina which in turn was supported by a fairly thick layer of collagen fibrils (arrows, Fig. 5) containing attenuated processes of fibroblasts. The basal plasma membrane was almost always straight, while the apical membrane was thrown into a few short and blunt microvilli (Figs. 5, 18). The lateral plasma membrane was also straight near the acinar lumen and demonstrated the usual junctional complexes common to most exocrine glands (Farquhar & Palade, 1963). However, the lateral membrane became more irregular towards the base of the cell, and there were interdigitations of villous projections from the adjacent cells (Figs. 5, 6, 12, 13). Small but well-developed desmosomes were present in this region maintaining, together with the irregular space created between villous projections, narrow intercellular canaliculi (Figs. 12, 13).

The Golgi apparatus was extensively developed and generally occupied a supranuclear position (Figs. 5, 6, 9). In any plane of section it showed at least a half dozen or more stacks of flattened lamellae along with the usual vacuolar components. Each stack of the Golgi complex was made up of 6–10 flattened saccules that showed dilated regions, particularly along the concave surface where secretory granules appeared to originate (Fig. 9). Lysosomes were frequently seen within this region (Figs. 6, 9, 11).

The secretory granules were mostly aggregated at the apical region of the cell, particularly in those acini that were loaded with a large number of granules. Granules were membrane-bound and remained well circumscribed, without signs of fusion. In cells that had relatively few secretory granules (Fig. 6) the granules accumulated in small patches at the apex. Frequently, a number of granules were found in the vicinity of the Golgi complex.

Elsewhere throughout the cytoplasm the rough-surfaced endoplasmic reticulum (RER) filled the basal and paranuclear regions of both granule-rich and granule-poor cells, except that in the latter there was a fair amount of the RER between the small patch of apical secretory granules and the Golgi complex (Fig. 6). The presence of the second zone of RER above the Golgi complex corroborated the light microscopic observations (Fig. 1).

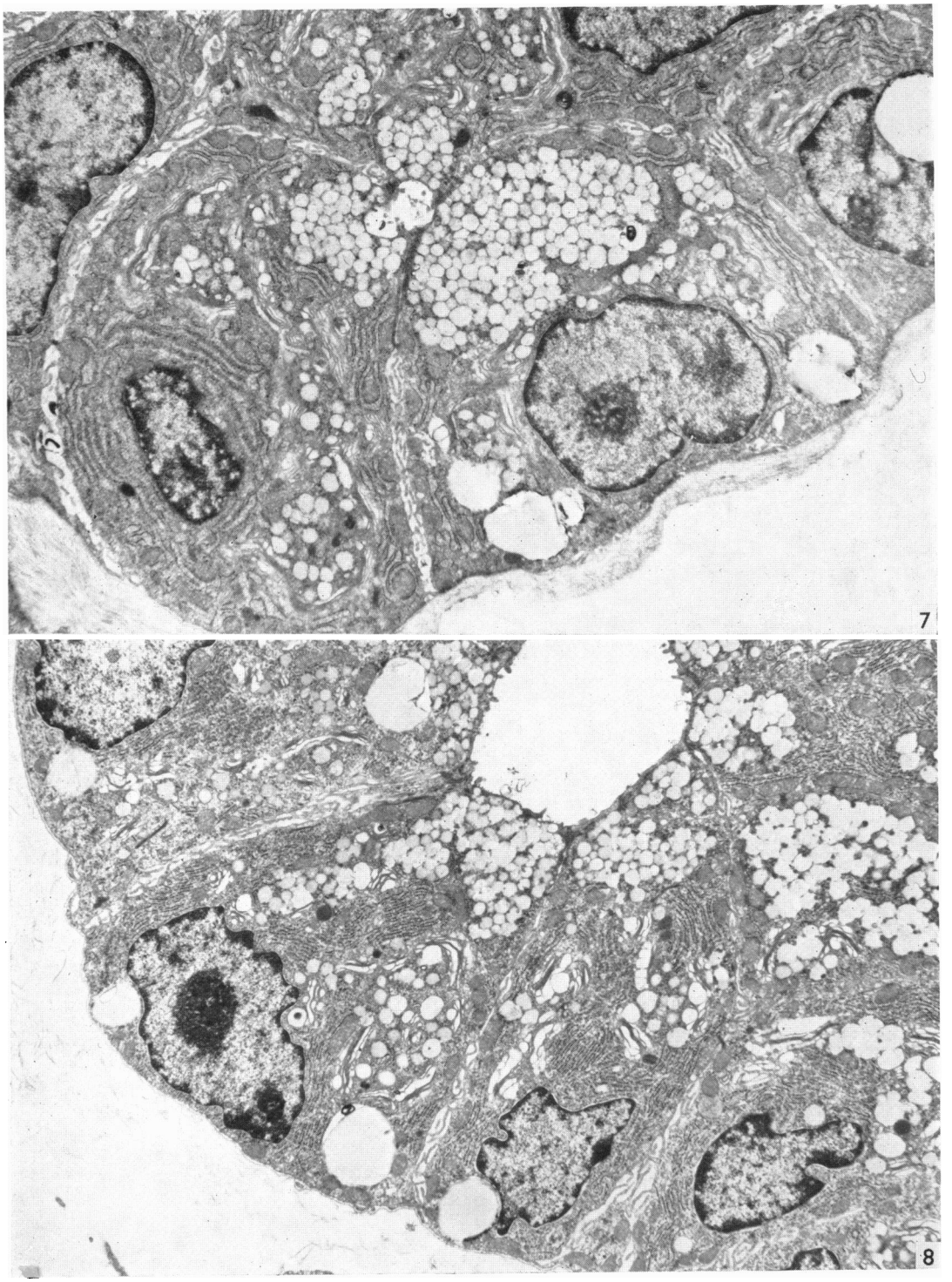


Fig. 7. A portion of the acinus from pair-fed animal. Secretory granules at the apex appear somewhat more irregular than normal. × 6000.

Fig. 8. A portion of the acinus from FUR-treated animal. Secretory granules show fusion and complex formations. × 6000.

Mitochondria of these cells were well developed and numerous. In addition to occupying the immediate vicinity of the Golgi complex and being interposed between elements of the RER, they were also present along the lateral border of the cells (Figs. 12, 13). They had a moderately electron-dense matrix and numerous cristae that extended the entire width of the organelle. Intermitochondrial granules were seldom present. Those mitochondria that were present along the lateral surface of the cell were frequently found in association with a desmosome (Figs. 12, 13).

The nucleus was basally located, having generally an ovoid contour with minor irregularities and occasional deep indentations (Fig. 6). The margination of the heterochromatin was limited to a narrow zone subjacent to the nuclear envelope. Occasionally, large nucleoli were found in association with small patches of heterochromatin (Figs. 6, 14) and demonstrated the usual structural complexity associated with metabolically active cells (Busch & Smetana, 1970).

Acinar cells of pair-fed control rats

The fine structure of acinar cells from the pair-fed control rats was similar to those of normal cells with certain exceptions. Unlike those from the normal gland the acinar cells of the pair-fed control rats always had a fairly large amount of apical granules. These granules tended to be somewhat more irregular in contour and included occasional large ones with aggregates of electron-dense materials (Fig. 7). The Golgi complex was extensive and resembled that of the normal glands, but the RER was somewhat reduced.

The most distinctive characteristics observed in the pair-fed control animal was the large number of lipid droplets in the basal region of the cytoplasm (Fig. 7). Here, autophagic vacuoles were also observed.

Acinar cells from FUR-treated rats

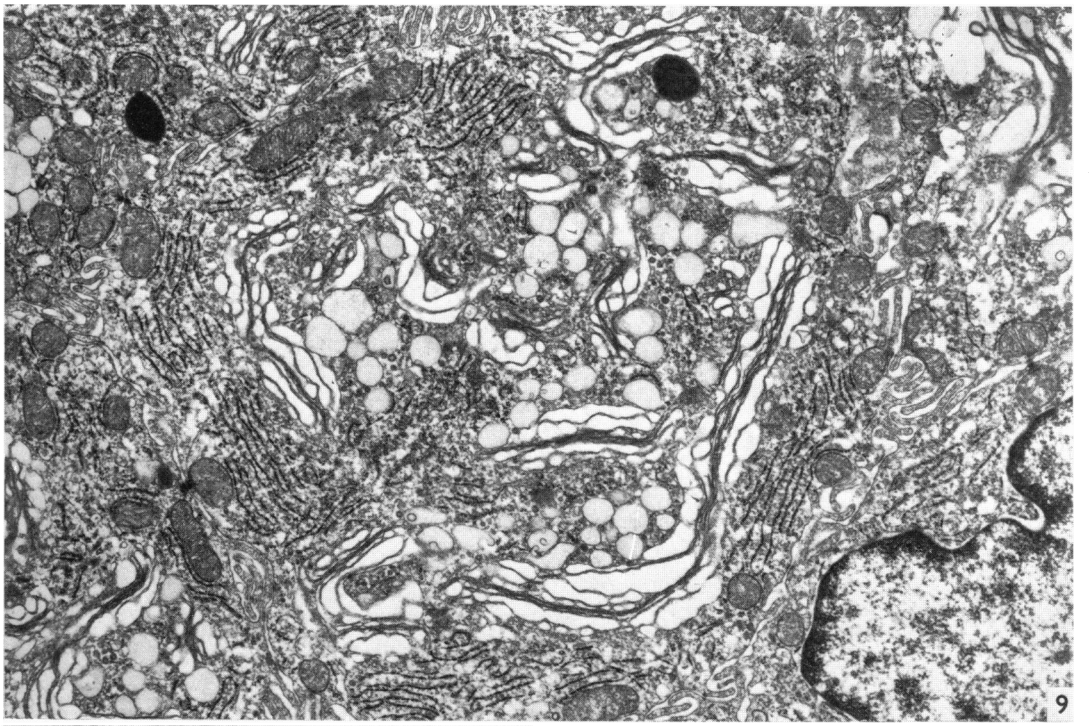
The appearance of acinar cells from FUR-treated rats varied in that comparatively normal looking acinar cells were interspersed among cells that showed severe degenerative changes. Secretory granules in FUR-treated cells were generally larger and more irregular in shape and tended to fuse with one another (Fig. 8). Such fused granules were not only present in the apical cytoplasm but could be found throughout the cytoplasm, particularly in association with the Golgi apparatus (Fig. 8).

The Golgi complex was made up of lamellar stacks that were shorter than those of normal or pair-fed control animals (cf. Fig. 10 with Fig. 9). In general, the stacks were made up of 6–10 lamellae that were tightly packed together (Fig. 10). The vesicles and vacuoles had a relatively electron-lucent interior. The number of mitochondria was reduced in FUR-treated glands.

Fig. 9. Extensive Golgi lamellae in an acinus from a normal gland. Note discrete granules with a homogenous matrix. × 11500.

Fig. 10. Segmented parts of the Golgi apparatus from FUR-treated animals associated with large irregular vacuoles with clear interior. The small stacks of lamellar elements are tightly packed. × 41000.

Fig. 11. Localization of AcPase activities in FUR-treated rat. Only four out of seven lysosomes shown demonstrate reaction products. × 51000.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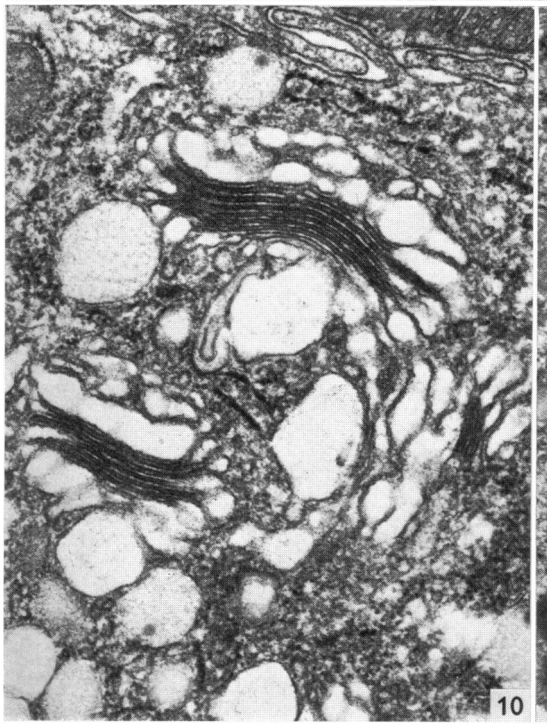
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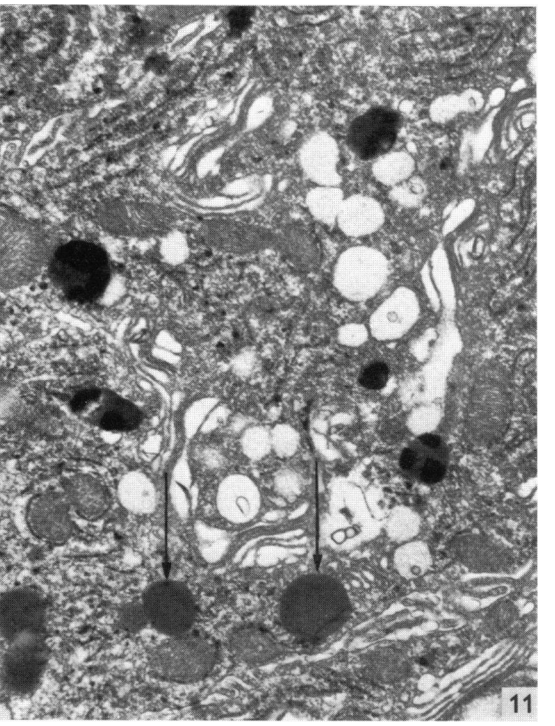
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The nucleus had a more jagged contour than that of the control or normal glands Fig. 8). Nucleoli were present in many cells: they were composed of segregated patches of nucleolonema and pars amorpha invested in a mass of nucleolus-associated heterochromatin (Fig. 15). As in the glands from pair-fed control animals, large lipid droplets were found at the base of the cell in large numbers. They were more frequent than in the pair-fed control and were occasionally found in the apical region of the cytoplasm as well (Fig. 8).

Localization of AcPase, UDPase and TPPase activities

In all cells AcPase activities were localized in dark osmophilic granules (primary lysosomes) and autophagic vacuoles (Fig. 11). The reaction products were confined to certain areas along the periphery of such granules. It is of interest that some of these granules were devoid of reaction product (note the two osmophilic granules arrowed at the bottom of Fig. 11).

UDPase and TPPase activities were both localized in the same region of the Golgi complex. Because of the more uniform localization of TPPase reaction products, illustrations in this article have been selected from the TPPase results. In general, TPPase activity was localized within two to three lamellae of the Golgi complex on the concave side of the Golgi complex (Fig. 16). Although the amount of reaction product was less in the experimental animals, there was no difference in localization. Furthermore, some of the granules, particularly those in the vicinity of the Golgi apparatus, gave a positive reaction for the enzyme (Figs. 16, 17).

A most intriguing observation was that, in addition to the presence of the lead deposits in the Golgi lamellae and vacuoles, similar deposits were formed along the luminal plasma membrane of the gland. They were located at the outer surface of microvilli (Fig. 18). They were also present in the valleys between microvilli and in the small vesicles that appeared to be associated with the cell surface (arrows, Fig. 18).

DISCUSSION

Normal and control glands

Glands of Brunner are of interest because they are confined to mammals and their function is uncertain. The early literature has been reviewed by Grossman (1958) and Cooke & Grossman (1966). Most investigators are of the opinion that the glands secrete an alkaline fluid containing mucin to protect the proximal duodenal mucosa against acid insult from gastric content. However, stomachs of platypus and echidna are aglandular and lined with stratified squamous epithelium (Krause, 1971), while the pH of gastric contents in the echidna does not fall below 6·7 even after a meal of

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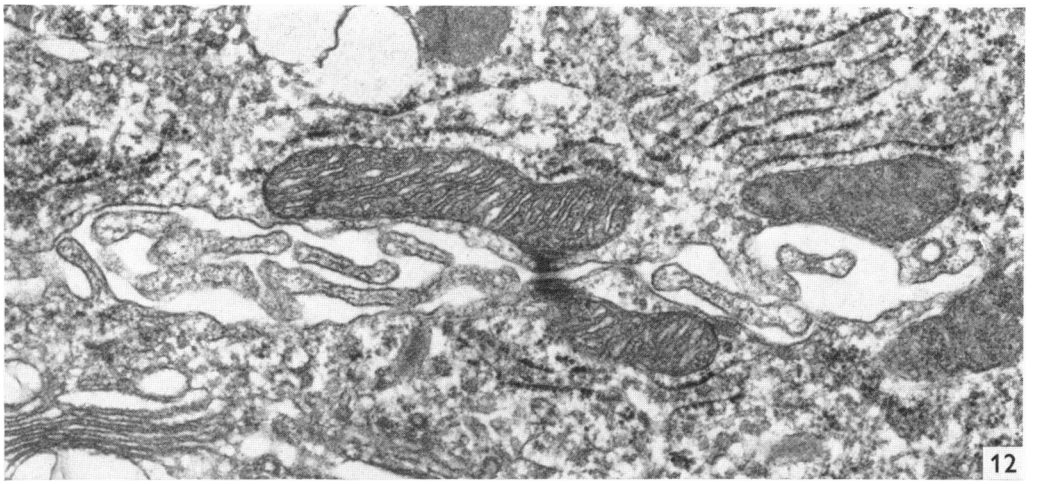
Fig. 12. Intercellular canaliculi, desmosomes, and the close association of mitochondria with the desmosomes are depicted. × 41000.

Fig. 13. Intercellular canaliculi, desmosomes, and the close association of mitochondria with the desmosomes are depicted. × 32000.

Fig. 14. A nucleolus from a normal gland. Note the large coiled nucleolonema. × 25000.

Fig. 15. A small nucleolus from a FUR-treated gland showing segregated and patchy appearance of nucleolonema. × 25000.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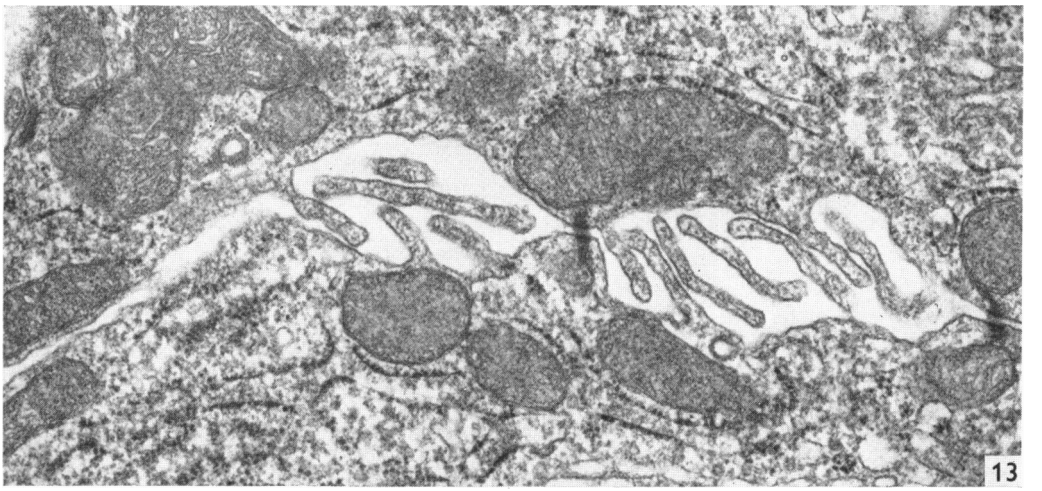
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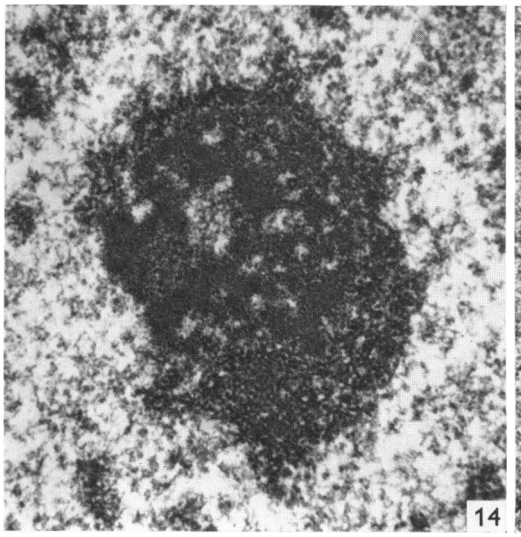
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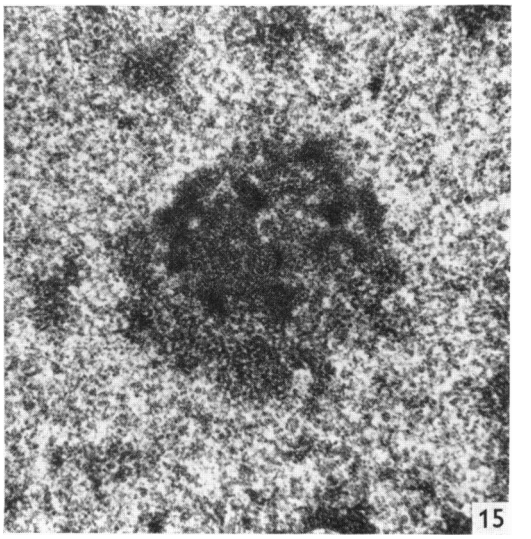
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termites (Griffiths, 1968). Therefore, an ant-acid role for Brunner's gland secretion seems doubtful in monotremes at least.

The cells in the guinea-pig (Cochrane *et al.* 1964) and rat (Leeson & Leeson, 1966) are reported to be typical mucous cells, whereas in the cat (Moe, 1960) and mouse (Friend, 1965) they include both serous and mucous types. In the rabbit (Leeson & Leeson, 1967) they are serous. The present ultrastructural observations on the rat indicate that, despite the electron-lucent and mucous nature of the granules, the cells in their general features belong to the serous type.

The close association of mitochondria with RER in the secretory cells of Brunner's glands has been observed by Friend (1965) in the mouse, by Krause & Leeson (1969) in the opposum, and by Krause (1971) in the platypus. Similar observations have been made in the rabbit and in man by Leeson & Leeson (1967, 1968). They suggested that the RER was involved in the synthesis of secretory materials. The same authors, however, did not find a close association of the two organelles in the rat (1966).

One of the more interesting points that has emerged from the present study is the intimate association of mitochondria with desmosomes along the lateral borders of secretory cells. It is tempting to speculate that the maintenance of desmosome junctions might be energy-dependent.

FUR effects

It is now clear that the administration of FUR results in a massive degeneration of cytoplasm which involves the various intracellular structures responsible for elaboration of secretory proteins. Past studies have included FUR effects on the exocrine pancreas (Martin, Levin & Kugler, 1969; Kugler, Levin, Martin & Sneddon, 1967), salivary glands (Kim & Han, 1970; Han, 1973; Kim & Han, 1972, 1973) and liver (Stenram, 1966). One of the consistent findings in exocrine glands is the breakdown of the Golgi complex. Changes in acinar cells of Brunner's glands are somewhat less striking, nevertheless certain changes are qualitatively similar to those which have been reported earlier, as evidenced by smaller stacks of lamellar elements and apparent fragility of the granule membranes.

The presence of a large number of lipid droplets in the experimental glands may indicate detrimental effects of the drug on the metabolism of the cells. However, similar cytoplasmic lipid granules in exocrine cells have been reported after injuries of various kinds (Han, 1967; Stenram, 1966; Han, 1973). It should also be emphasized that lipid granules are present in cells from pair-fed control animals. Thus, the lipid granules in FUR-treated glands seem to be a non-specific secondary effect of the drug.

The increase in number of primary lysosomes may indicate that they are involved in the breakdown of segregated cytoplasmic components. It is also possible, however, that a suppression of secretory activity itself may indirectly lead to an accumulation

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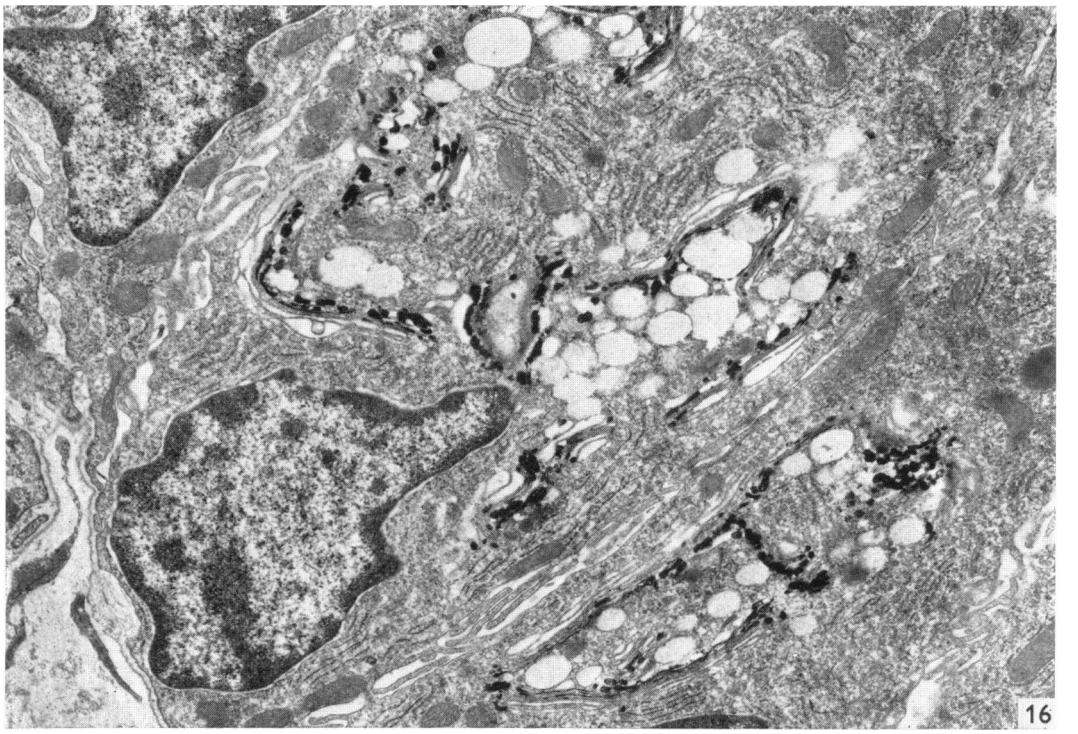
Fig. 16. Normal gland. TPPase activity is localized in the distal two to three Golgi cisternae. × 12000.

Fig. 17. As well as in the Golgi lamellae, TPPase activity is localized in the newly formed secretory granules associated with the Golgi region. × 50000.

Fig. 18. In some areas, TPPase activity is localized along the cell surface, particularly in association with the microvilli and small vesicles (arrows). × 50000.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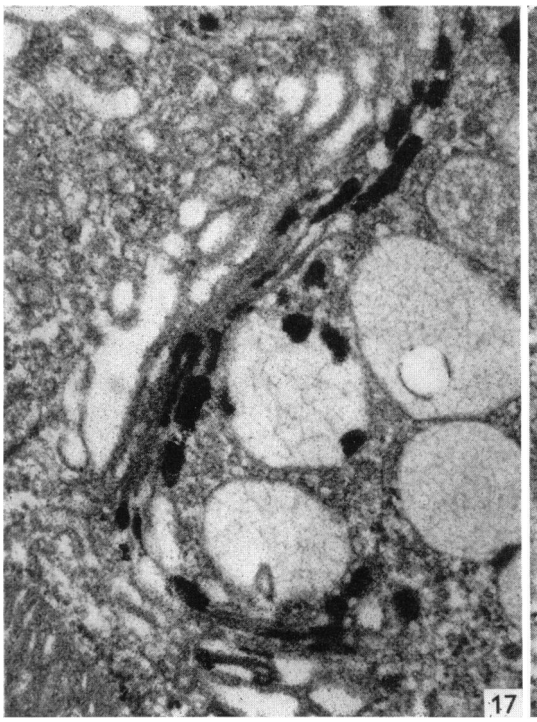
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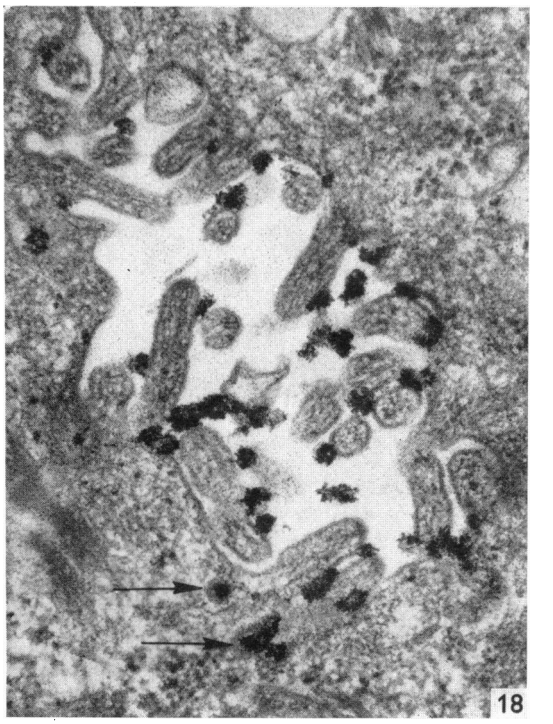
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of primary lysosomes because of their reduced utilization in granule maturation (Hand, 1973). The effect that acid phosphatase activity is not present in some of the primary lysosomes suggests that *an increase in the number of lysosomes seen in regular electron micrographs should not be construed as necessarily indicating increased lysosomal activity.*

Cytochemical evidence for participation of Golgi lamellae in secretory granule formation

Despite the widely accepted notion that the Golgi complex is important in the packaging of secretory granules, varying opinions exist as to the manner in which different parts of the Golgi complex may be involved in this process. Jamieson & Palade (1967, 1971) believe that the secretory granules in the pancreas are produced in condensing vacuoles from transport vesicles originating in the RER. Novikoff *et al.* (1971) recognized in neurons of dorsal root ganglia the presence of an acid phosphatase-rich inner surface (GERL) which they consider to be a special component of the Golgi complex functionally independent of the diphosphatase-rich surface of the complex. Work from Leblond's laboratory which has involved extensive radioautographic studies of the Golgi complex in a variety of mucous secreting cells has shown the incorporation of many simple sugars into the Golgi lamellae (Peterson & Leblond, 1964; Neutra & Leblond, 1966*a*, *b*; Weinstock & Leblond, 1971).

It is quite possible that the Golgi complex may be functioning in different ways in different types of cells under different physiologic conditions. Inasmuch as the Golgi complex is observed in most, if not in all, cells its involvement in secretory processes could not be the exclusive function of the Golgi apparatus. However, it is significant that the nucleoside diphosphatase activities are localized in the lamellar elements which have been identified as the incorporation site of simple sugars. While the UDPase or other nucleoside diphosphatase activities are not directly involved in the synthesis of the polysaccharides, they do influence the amount of nucleotide intermediates necessary for polysaccharide synthesis.

The TPPase, a cocarboxylase, has not been implicated in the synthesis of carbohydrate moieties. Despite this it has been repeatedly found to exist in the same regions of the Golgi complex where UDPase activities are located (Essner & Novikoff, 1962; Novikoff *et al.* 1971; Kim & Han, 1973). Assuming that the coincidental presense of TPPase and UDPase activities in the distal elements of the Golgi lamellae can be accepted as indicative of the involvement of these membranes in polysaccharide synthesis, one is tempted to speculate that *the presence of their activities in the distal membranes, in the newly formed secretory granules and in the apical membranes of the secretory cells may represent the functional flow of the lamellar membranes to the apical plasma membrane via the secretory granule.* It should be emphasized, however, that even if there is a flow of membrane as suggested by this study between the Golgi lamellae and the apical plasma membrane of acinar cells, there are distinct differences in the biochemical makeup of the various membrane components of secretory cells (Essner & Novikoff, 1962; Meldolesi, Jamieson & Palade, 1971*a*, *b*, *c*).

The presence of enzyme activities in the small vesicles near the plasma membrane supports the recently advocated concept that secretory cells retrieve the extramembrane added to their luminal surface during exocytosis (Hand, 1973).

SUMMARY

Effects of a pyrimidine analogue, 5-fluorouracil (FUR), have been studied by electron microscopy and by electron microscopic cytochemical techniques. Previous studies have demonstrated that rats show serious gastrointestinal disturbances 5 days after 3 daily injections of FUR (50 mg/kg). The present investigation demonstrates that Brunner's glands under the same conditions suffer certain cytological changes involving the Golgi apparatus, where a notable reduction in the number of Golgi stacks is observed. The vacuolar components in the Golgi complex appear empty. Cytochemical localizations of uridine diphosphatase and thiamine pyrophosphatase activities, however, are normal. The reaction products are localized in the distal two or three lamellae of the Golgi stack and within the secretory granules nearby. In addition reaction products are present along the apical plasma membrane on the luminal side, suggesting a possible movement of these membranes from the Golgi stack, via secretory granules, to the apical plasma membrane.

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